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TECHNICAL MANUSCRIPT 485

QUANTITATIVE ASSAY OF INTERFERON BY THE IMMUNOFLUORESCENT CELL-COUNTING TECHNIQUE

Edmund H. Kozikowski
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NOVEMBER 1968

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QUANTITATIVE ASSAY OF INTERFERON BY THE IMMUNOFLUORESCENT CELL-COUNTING TECHNIQUE

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Project 1T013001A91A

November 1968

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

A rapid, quantitative assay of interferon was developed, based on the reduction of the number of virus-infected cells by dilutions of interferon. Infected cells were enumerated by fluorescent cell-counting 24 hours after challenge of interferon-treated McCoy cover-slip cell monolayers. A quantitative linear relationship between the reduction of fluorescent cells and dilutions of interferon was demonstrated by probit transformation of inhibition percentages. Yellow fever virus, selected as the standard challenge virus, was the most sensitive of four viruses tested to the action of interferon prepared in McCoy cell cultures.

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I. INTRODUCTION

Although numerous systems have been devised for the assessment of interferon activity, the majority are adaptations of established virus assay techniques based on the reduction of cytopathic effects. The various interferon assay methods that are currently employed have been described by Finter[1] along with comments on their advantages and limitations. Recently, immunofluorescent cell-counting assays have been developed for a number of viruses;[2-8] these assays are based on the enumeration of cells containing viral antigen, are not dependent on viral cytopathogenicity, involve only one cycle of infection, and are direct (each fluorescent cell is the result of infection by one viable virus particle). High sensitivity, precision, ease of performing replicate determinations, and unusual rapidity (less than 24 hours) are outstanding features of the assays. In view of these advantages, the feasibility of extending the principle of the technique for the assay of interferon was investigated.

II. MATERIALS AND METHODS

A. VIRUSES

Vaccinia virus, IHD strain, previously grown upon the chorioallantoic membrane of embryonated chick eggs, was passed twice in McCoy cells. The virus suspension, assayed by the fluorescent cell-counting technique, contained 1.3 x 10^9 cell infecting units (CIU) per ml.

A suspension of the Asibi strain of yellow fever virus was obtained from an infected rhesus monkey that had been inoculated intraperitoneally with 2 x 10^4 CIU of virus and bled 3 days later. Infectious plasma contained 3 x 10^8 CIU of virus per ml.

Suspensions of the Borg strain of psittacosis agent and the Trinidadian donkey brain strain of Venezuelan equine encephalomyelitis (VEE) virus were prepared from infected McCoy cell cultures. These virus suspensions were distributed in 1-ml portions into glass vials and stored at -60 C.

B. CELL LINES AND CULTIVATION

The established McCoy cell line, derived from human synovial tissue,[9] was used for the assay of virus infectivity. Nutrient medium for the cell line consisted of medium 199 containing 0.5% (w/v) lactalbumin hydrolysate, 10% calf serum, and 50 μg of streptomycin per ml and 75 μg kanamycin per ml. Cells were maintained in medium 199 with 5% calf serum. For interferon and virus assays, cells were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). A 1-ml amount of cell suspension containing 1×10^5 to 3×10^5 cells was introduced onto cover slips that were then incubated at 35 C for 24 hours, or until complete monolayers were formed. Cover-slip cell cultures were washed twice with 2 ml of maintenance medium prior to the addition of virus inoculum or interferon.

C. PREPARATION OF INTERFERON

For induction of interferon from cell cultures, 5-ml quantities of vaccinia virus were held in Vycor* test tubes (15 by 125 mm) and irradiated for 10 minutes with ultraviolet light from two 15-watt G.E. germicidal lamps at a distance of 2 inches. Plastic tissue culture flasks** (75 cm^2) containing McCoy cell monolayers were exposed to 3 ml of undiluted irradiated vaccinia virus at 35 C for 4 hours. The cell sheets were washed twice, incubated again at 35 C with 5 ml of maintenance medium, and the fluids were harvested 24 hours later. Interferon preparations were centrifuged at 105,000 x g for 90 minutes to remove virus and cell debris. The supernatant fluids were dialyzed against HCl-KCl buffer, pH 2.0, at 4 C for 24 hours, then against two changes of Earle's BSS, pH 7.1, at 4 C for 24 hours. The dialyzed fluids were distributed into 17 by 64 mm capped plastic test tubes and stored at -60 C until assayed for interferon activity.

That preparations possessed biological and physical properties similar to those described for viral interferon was established by their nondialyzability, stability at pH 2.0, nonsedimentation at 105,000 x g for 90 minutes, sensitivity to trypsin, stability at 56 C for 1 hour, cell species specificity, nonspecificity for viruses, persistence of inhibitory effect after washing treated cells, and absence of any virucidal effect.

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* Corning Glass Works, Corning, N.Y.

** Falcon Plastics, Los Angeles, Calif.

D. INTERFERON ASSAY

Serial twofold dilutions of interferon suspension prepared in maintenance medium were introduced in 0.5-ml volumes directly into vials containing cover-slip cultures of McCoy cells and incubated at 35 C for 24 hours. The assays were carried out in triplicate. Control cultures contained spent medium from noninfected McCoy cell cultures prepared in the same manner as the interferon preparations. After incubation, the cover-slip cultures were washed twice with maintenance medium and then challenged with approximately 7×10^3 CIU of virus in 0.2-ml volumes. Attachment of virus to cells was carried out with the aid of centrifugal force. The psittacosis agent and vaccinia virus were attached to cells at centrifugation speeds of 500 x g for 15 minutes; yellow fever and VEE viruses were attached at centrifugation speeds of 19,642 to 29,432 x g for 15 minutes. Following attachment, 1 ml of appropriate maintenance medium was added to vials, which were then incubated at 35 C in accord with prescribed periods for each virus assay (18 to 24 hours). Details of the procedures have been recorded elsewhere.[7,10-12] Cover-slip cultures were then rinsed twice with cold PBS, fixed with acetone (-60 C), and either prepared immediately for immunofluorescent staining and counting or stored at -60 C.

The direct fluorescent-antibody technique was employed to demonstrate immunofluorescence in infected cells. Fixed cell cultures were washed once with PBS and stained with conjugate for 30 minutes. The cover-slip cell cultures were rinsed in two changes of PBS to remove excess conjugate and then mounted in semipermanent medium.[13]

E. FLUORESCENCE MICROSCOPY

Cover-slip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator (model 645; Corning No. 5840 and Schott BG-12 exciter filters, and an E.K. No. 2A barrier filter).

F. FLUORESCENT CELL-COUNTING AND CALCULATIONS

At 430 times magnification with the optical system employed, 1,064 microscopic fields were contained in the area of a 15-mm cover slip. For each cover-slip cell culture, 50 microscopic fields were examined for fluorescent cells. To calculate the number of cell-infecting units of virus per milliliter, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to milliliters).

G. DETERMINATION OF INTERFERON ACTIVITY

The activity of the interferon preparations was determined by plotting the per cent reduction of fluorescent cell counts against the logarithm of the corresponding interferon dilutions. The resulting dose-response curve was sigmoid, but a straight-line relationship was obtained by probit transformation of reduction percentages.[14] The interferon titer was determined from the interferon dilution that intersected the 50% reduction value (probit = 5) on the dose-response line. Its reciprocal was expressed as interferon units per 0.5 ml.

III. RESULTS

A. SENSITIVITY OF DIFFERENT VIRUSES TO INTERFERON

A standard challenge agent was selected for the assay system by testing four viruses for inhibition by interferon. Serial twofold dilutions of interferon were inoculated onto McCoy cover-slip cultures, which were then incubated at 35 C for 24 hours. Cell cultures were challenged with either the psittacosis agent or vaccinia, VEE, or yellow fever viruses. A linear dose-response relationship was established for each challenge virus by plotting probits of per cent reduction of infected cells against the logarithm of the interferon dose (Fig. 1). Yellow fever virus was more sensitive to the action of the interferon preparation than the other test viruses and was selected as the standard challenge agent. Its sensitivity was 119, 11, and 6 times that of the psittacosis agent, vaccinia virus, and VEE virus, respectively.

B. DURATION OF INTERFERON CONTACT WITH CELLS BEFORE VIRUS CHALLENGE

To exert maximal antiviral effect, an interval of interferon contact with host cells is required before the addition of challenge virus.[15,16] This period was determined by adding interferon (diluted to give approximately 80% inhibition) simultaneously with virus and at 4, 7, 12, 20, and 24 hours prior to virus challenge. Table 1 indicates that some inhibition of viral activity was detected when interferon was in contact with cells for 12 hours. Maximal inhibition was achieved, however, when interferon had been in contact with cells for 24 hours prior to virus challenge.

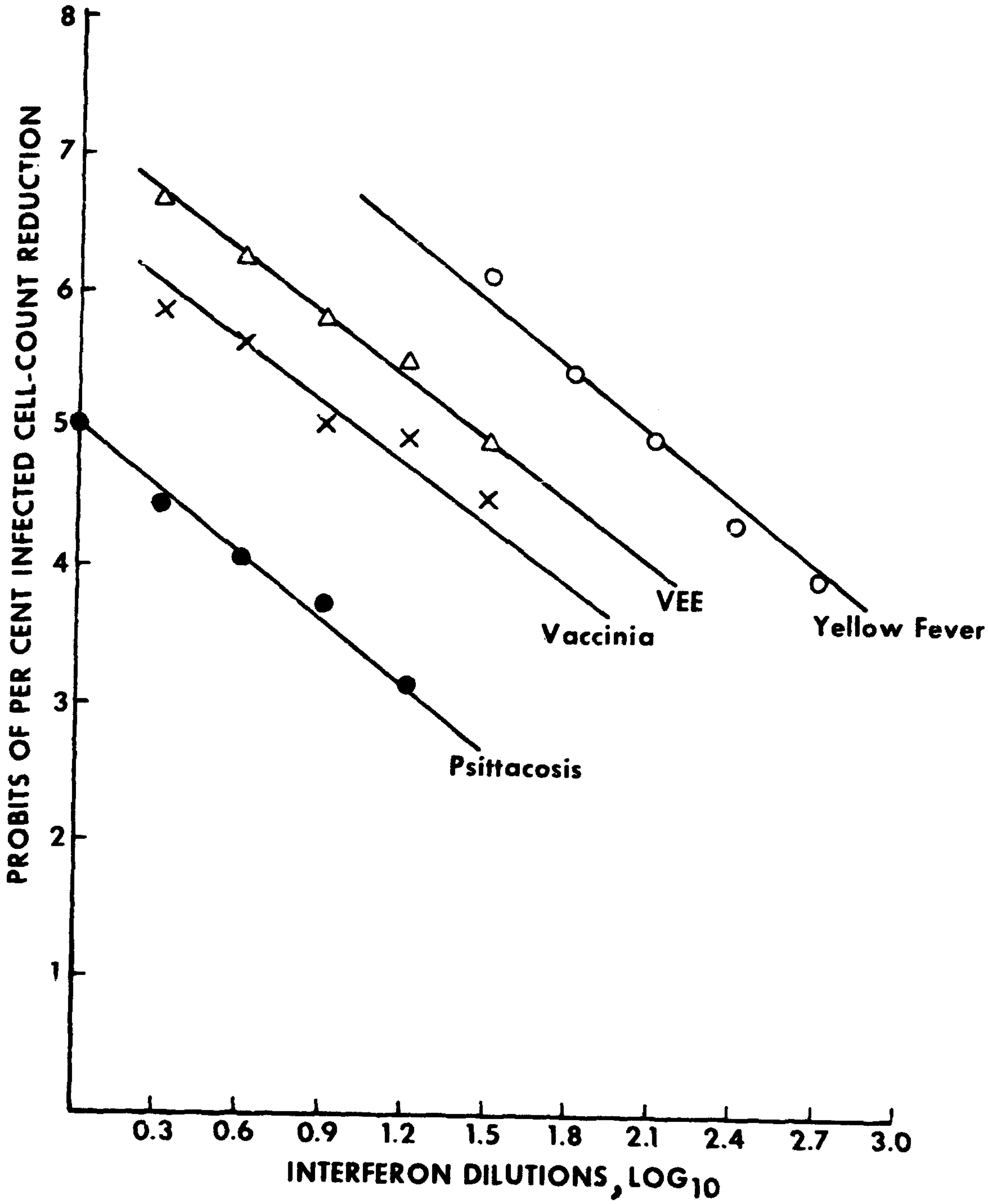

FIGURE 1. A Comparison of the Sensitivity of Psittacosis Agent and Vaccinia, VEE, and Yellow Fever Viruses to a Preparation of McCoy-Cell Interferon.

TABLE 1. DURATION OF INTERFERON CONTACT WITH CELLS AND ITS EFFECT ON PER CENT REDUCTION OF INFECTED CELL COUNTS

Duration of Interferon Contact, hours[a]	Infected Cell Counts		% Reduction
	Controls	Treated	
0	191	191	None
4	252	290	None
7	138	183	None
12	121	72	40.5
20	116	34	70.7
24	182	23	87.3

a. Virus and interferon inoculated simultaneously.

C. EFFECT OF CHALLENGE DOSE ON INTERFERON TITER

A preparation of McCoy-cell interferon was assayed by employing high and low doses of yellow fever virus to determine the effect of the challenge dose on the interferon titer. Challenge doses employed were 2.0×10^3 and 1.2×10^4 CIU/0.2 ml of inoculum. Table 2 shows that there was no significent change in interferon titers when two doses of challenge virus, differing by approximately 1.0 log unit in concentration, were employed.

TABLE 2. EFFECT OF YELLOW FEVER VIRUS CHALLENGE DOSE ON THE TITER OF A McCOY-CELL INTERFERON PREPARATION

Challenge Dose, CIU/0.2 ml	Interferon Titer, units/0.5 ml
2.0×10^3	125[a] 100
1.2×10^4	106 100

a. Two independent titrations for each challenge dose.

D. PRECISION OF THE INTERFERON ASSAY

An estimate of the precision of the assay was obtained from seven determinations of the same interferon preparation. Cover-slip cultures were treated with 0.5-ml volumes of twofold interferon dilutions and then challenged with a standard quantity of yellow fever virus inoculum. Table 3 shows that interferon titers ranged from 100 to 126 units per 0.5 ml with a mean titer of 111 units. The standard deviation (SD) was ±10.6. Expressed as a percentage of the mean, the SD was 9.5%.

TABLE 3. PRECISION OF INTERFERON TITER BY THE FLUORESCENT CELL REDUCTION ASSAY

Assay	Interferon Titer,[a/] units/0.5 ml
1	112
2	100
3	126
4	106
5	112
6	125
7	100
Mean	111
Standard deviation	±10.6
SE of mean	±4.0

a. Reciprocal of the interferon dilution reducing the infected cell count to 50% of the controls.

IV. DISCUSSION

The application of an immunofluorescence technique to assess the protective status afforded to cells against challenge virus by their prior treatment with interferon has been reported previously.[17,18] In principle, the immunofluorescent assay of interferon described in the present report is similar to the former in that the reduction of cells containing fluorescent viral antigen serves as the indicator of interferon activity. In contrast to the earlier procedure, however, the current interferon assay is based on a standardized and highly quantitative virus assay system that provides for efficient and effective virus-cell interaction that results in maximum sensitivity and precision. The ability to detect and to assay interferon preparations with accuracy is a reflection of this new assay system.

Of four viruses employed to challenge interferon-treated cells, yellow fever virus was the most sensitive to the protective activity of interferon. The comparison attains greater validity because the assessment procedure was carried out with a standardized fluorescent cell-counting technique for each agent and with the same established cell line. Dose-response curves formed parallel straight lines when the probits of fluorescent cell reduction percentages were plotted against the log of the interferon dilutions. The relative sensitivity of the virus agents or estimate of interferon titer is obtained directly by measuring the distance between lines along the axis of the 50% probit transformations of fluorescent-cell reduction percentages. The selection of yellow fever virus as the challenge agent in this study does not preclude the possibility that other viral agents may be equally as sensitive or more sensitive for the detection and measurement of interferon potency. A more reliable comparison among different viral agents is invited with the increased number of available fluorescent cell-counting assays. With yellow fever virus as the challenge agent, the immunofluorescent assay of interferon was successfully employed to detect and estimate the induction of interferon by Coxiella burnetii.[19] It was also sensitive enough to detect interferon induced by adenovirus-4 from guinea pig leukocytes.*

The dose of the challenge virus over a range of approximately 1.0 log unit did not significantly affect the interferon titer. That the titer of an interferon preparation is independent of the dose of the challenge virus is in agreement with the findings of others.[14,20,21] Of the many interferon assays in use, relevant data on the precision of the assays has been curiously neglected.[1] Published data show: (i) an SD of 0.267 $\log_{10}$ for 14 determinations by the plaque-agar overlay method,[14] (ii) an SD of 0.12 $\log_{10}$ units for 15 determinations by the inhibition-hemadsorption method,[1] and (iii) an SD of 0.22 $\log_{10}$ units for six assays by the plaque-fluid overlay method.[14] By the immunofluorescent cell-reduction assay of interferon described here, the SD was 0.04 for seven determinations,

* Kozikowski, E.H., unpublished data.

which is identical to that cited for the assay of yellow fever virus by fluorescent cell-counting.[18] In general, the immunofluorescent assay of interferon based on the reduction of yellow fever virus - infected cells adequately fulfills most, if not all, of the proposed criteria for interferon assays.[1]

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13. ABSTRACT

A rapid, quantitative assay of interferon was developed, based on the reduction of the number of virus-infected cells by dilutions of interferon. Infected cells were enumerated by fluorescent cell-counting 24 hours after challenge of interferon-treated McCoy cover-slip cell monolayers. A quantitative linear relationship between the reduction of fluorescent cells and dilutions of interferon was demonstrated by probit transformation of inhibition percentages. Yellow fever virus, selected as the standard challenge virus, was the most sensitive of four viruses tested to the action of interferon prepared in McCoy cell cultures.

14. Key Words

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*Assay
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